Clinical, Experimental and Pathophysiological effects of Yaq-001, a Non-absorbable, Gut-restricted Adsorbent in Models and Patients with Cirrhosis

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Conflict of Interest

Rajiv Jalan is the inventor of OPA, which has been patented by UCL and licensed to Mallinckrodt Pharma. He is also the founder of Yaqrit Discovery, Hepyx Limited (spin out companies from University College London), and Cyberliver. He has research collaborations with Yaqrit Discovery. Yaq-001 was licensed by Yaqrit Ltd. from UCL. JRM has received consultancy fees from EnteroBiotix and Cultech, and speaker fees from Falk Forum.

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Authors' contributions

RJ, FA, JL, JM, ND - contributed to the conception and design of the study. SS and GI contributed to the conception and design of the *in vitro* studies.   RJ, ND, FA - provided

12

Abstract

Objective: Strategies to target bacterial translocation in cirrhosis are limited to antibiotics with risk of antimicrobial resistance. This study explored the therapeutic potential of a non-absorbable, gut-restricted, engineered carbon bead adsorbent, Yaq-001 in models of cirrhosis and acute-on-chronic liver failure (ACLF) and, its safety and tolerability in a clinical trial in cirrhosis.

Design: Performance of Yaq-001 was evaluated *in vitro*. Two-rat models of cirrhosis and ACLF, [4-week, bile duct ligation (BDL)], receiving Yaq-001 for 2-weeks; and two-mouse models of cirrhosis [6-week and 12-week carbon tetrachloride (CCL4)] receiving Yaq-001 for 6-weeks were studied. Organ and immune function, gut permeability, transcriptomics, microbiome composition and metabolomics were analysed. Effect of fecal water on gut permeability from animal models were evaluated on intestinal organoids. A double-blind, randomized, placebo-controlled clinical trial in 28-cirrhosis patients, administered 4-gram/day Yaq-001 for 3-months was performed.

Results*:* Yaq-001 exhibited rapid adsorption kinetics for endotoxin. *In vivo*, Yaq-001 reduced liver injury, progression of fibrosis, portal hypertension, renal dysfunction and mortality of ACLF animals significantly. Significant impact on severity of endotoxemia, hyperammonemia, liver cell death, systemic inflammation and organ transcriptomics with variable modulation of inflammation, cell death and senescence in the liver, kidneys, brain and colon was observed. Yaq-001 reduced gut permeability was noted in the organoids and impacted positively on the microbiome composition and metabolism. Yaq-001, regulated as a device met its primary end point of safety and tolerability in the clinical trial.

Conclusions: This study provides strong pre-clinical rationale and safety in patients with cirrhosis to allow clinical translation.

14

Significance of this study

What is already known on this topic?

Current strategies to target bacterial translocation in cirrhosis are limited to antibiotics with risk of resistance. Yaq-001 is an insoluble, non-absorbable, non-antibiotic, engineered carbon bead of tailored porosities, which works as an adsorbent in the gut and is completely excreted after oral administration.

What this study adds?

1. Yaq-001 rapidly adsorbs endotoxin, ammonia and bile acids without influencing bacterial growth kinetics *in vitro*.

2. Yaq-001 reduces mortality of ACLF animals and impacts positively on markers of gut permeability, liver injury, portal pressure, brain and kidneys in animal models of cirrhosis and ACLF.

3. Yaq-001 administration was associated with positive impact on the composition of the gut microbiota, reduction in severity of endotoxemia and ammonia, severity of inflammation, liver cell death, signaling pathways and LPS sensitivity.

4. In animal models of liver fibrosis and cirrhosis, it reduces the severity of liver injury and hepatic fibrosis.

5. Enhanced permeability of intestinal organoids following incubation with fecal water from cirrhosis animals is prevented by treatment of the cirrhosis animals with Yaq-001.

6. In a Phase 2, double-blind, randomized, controlled clinical trial of Yaq-001 versus placebo in patients with cirrhosis, Yaq-001 was found to be safe and well tolerated providing evidence of clinical translatability.

How this study might affect research, practice or policy?

The data provide the pre-clinical rationale and clinical safety to proceed to the next phase of clinical trials in patients with cirrhosis aiming to prevent the occurrence of complications.

INTRODUCTION

Gut dysbiosis and gut-derived bacterial ligands, in particular endotoxin, drive a dysregulated inflammatory response, which has been implicated in the development of cirrhosis and its complications such as sepsis, spontaneous bacterial peritonitis, renal dysfunction and hepatic encephalopathy[1-3]. This dysregulated inflammatory response is also central in the development of acute-on-chronic liver failure (ACLF)[4]. Markers of bacterial translocation such as endotoxin and bacterial DNA have been shown to be associated with complications of cirrhosis and diminished survival highlighting their pathogenic importance[5,6,7]. The microbiome in cirrhosis is characterized by reduced diversity and abundance of autochthonous bacteria[1]. Whilst antibiotics have been shown to impact positively on complications of cirrhosis, their use is associated with antibiotic resistance[8,9]. Furthermore, antibiotics reduce bacterial diversity rendering the microbiome less resilient.

One of the consequences of bacterial translocation in cirrhosis is that the endotoxin-sensing pathways in different organs are known to be primed resulting in heightened susceptibility to organ injury[3,10]. Adsorption of free endotoxin without exerting direct effects on bacterial growth kinetics, therefore has the potential to attenuate susceptibility to organ injury without producing the deleterious effects on the microbiome. Considering this, we developed a synthetic non-absorbable, non-antibiotic, endotoxin sequestrant and generated the hypothesis that this may be a novel therapeutic strategy to restore the microbiome, prevent bacterial translocation, systemic inflammation progression of fibrosis and cirrhosis complications. Yaq-001 is a non-absorbable, highly engineered, activated carbon of multiple porosities tailored to the micro (<2nm) and meso-macroporous (30-200 nm) range and high surface area[11-13]. These properties confer a high adsorptive capacity for larger biologically relevant molecules such as bacterial toxins in addition to smaller intraluminal targets. The most closely associated experimental oral adsorbent is AST-120, a microporous carbon bead, which has not been shown to be efficacious in patients with hepatic encephalopathy[14].

417   In this study, we sought to determine the adsorptive capacity of Yaq-001 and its effect on
418   bacterial growth kinetics in *in vitro* studies. We then evaluated the *in vivo* biological effects
419   of Yaq-001 in four animal models representing characteristics of cirrhosis and ACLF. We
420   studied the effects of Yaq-001 on measures of multiorgan function, systemic and portal
421   hemodynamics, immune function, multiorgan transcriptomics and microbiome
422   composition. Finally, we performed a Phase 2 equivalent double-blind, multicenter,
423   randomized, placebo-controlled clinical trial to assess the safety and tolerability of Yaq-
424   001 in patients with decompensated cirrhosis.
425

METHODS

Methodological details are described in **Supplementary section**.

Functional and Structural Characteristics of Yaq-001

Adsorption of biomolecules of varying molecular weights (albumin, myoglobin, and caffeine) was evaluated. Bacterial growth was studied for *Staphylococcus aureus (S. aureus)* and *Escherichia coli (E. coli)*. Scanning electron microscopy was performed to characterise the beads and pore size distribution was assessed using mercury porosimetry.

Studies in animal models

Study design

These studies aimed to characterize the therapeutic potential of Yaq-001 in rats and mice to define its role in prevention of occurrence of cirrhosis, progression of cirrhosis and occurrence of ACLF (**Fig.S1 and Fig.S2**).

Animal models

Four-week bile-duct ligation model of advanced fibrosis

    a. Cirrhosis: Sham (n=36); Sham-Yaq-001 (n=30); BDL (n=37); BDL-Yaq-001 (n=44).

    b. *Prevention of ACLF:* Sham-LPS (n=9); Sham-LPS-Yaq-001 (n=10); BDL-LPS (n=16); BDL-LPS-Yaq-001 (n=12).

Yaq-001 (0.4 g/100g body weight per day) was administered for 2-weeks prior to sacrifice. At the time of sacrifice, mean arterial pressure (MAP) and portal pressure were measured.

Carbon tetrachloride treated model of cirrhosis

    c. Advanced fibrosis and early cirrhosis (CCl$_4$ for 6-weeks): Control (n=6); Control-Yaq-001 (n=6); CCl$_4$ (n=12); CCl$_4$-Yaq-001 (n=12).

    d. Advanced cirrhosis (CCl$_4$ for 12-weeks): Control (n=6); Control-Yaq-001 (n=6); CCl$_4$ (n=12); CCl$_4$-Yaq-001 (n=12).

457

458   Yaq-001 (0.4 g/100 g body weight per day) was administered from 0-6 weeks in the 6-
459   week model and from 6-12 weeks in the 12-week model.

460

461   ***Collection and analysis of bio-samples***

462   Blood, stool and tissues samples were collected for later analysis. Portal venous blood
463   was collected where possible. Peripheral blood cells and Kupffer cell reactive oxidant
464   species (ROS) were measured. Hematoxylin-Eosin (H&E), Picrosirius Red (PSR) staining
465   and TUNEL stains were performed in liver tissues. The mRNA in different organs was
466   analyzed by using nSolver4.0 software (NanoString Technologies). To define effect on
467   the microbiome, 16s microbiome study was performed. To determine the effect of Yaq-
468   001 on modulating metabolism, urinary ^{1}H-NMR analysis was performed.

469

470   **Assessment of gut permeability in Intestinal organoids**

471   Permeability of mouse intestinal organoids were detected using established protocols[15].
472   Fecal water generated from the stools obtained from the four groups of 6-week CCl4 mice
473   were incubated with the organoids as described previously. Permeability of the organoids
474   were assessed.

475

476   **Clinical trial of Yaq-001 versus placebo, CARBALIVE-SAFETY study**

477   ***Study Design***

478   The CARBALIVE-SAFETY clinical trial was a first in man, multicenter, double blind
479   randomized, placebo-controlled trial of oral Yaq-001 in stable decompensated cirrhosis.
480   Details of the study protocol is available in the Supplementary section (**Fig.S3**). As Yaq-
481   001 is regulated as a device, it followed both ISO standards and ICH-GCP guidance.
482   Informed consent was obtained from each patient. The study was closely monitored and
483   overseen by an independent data safety monitoring board (NCT03202498).

484

485   Study design is described as **Fig.S4**. The primary end point was assessed at 12-weeks.
486   Blood and stool samples were taken at the time of randomization, 4-weeks and 12-weeks
487   for assessment of some of the secondary and exploratory end points. Safety

488 assessments were performed on weeks 1, 4, 8 and 12 and comprised a physical
489 examination, clinical laboratory tests, urinalysis, 12-lead ECG and an assessment of
490 reported and observed adverse events. ECGs were analyzed independently. Nutritional
491 status was assessed by the Royal Free Hospital Global Assessment tool at each safety
492 assessment together with micronutrient analysis at baseline, week 4 and 12. Vitamin B12,
493 A, D, E, folate, and K1 and, trace elements Copper, Zinc and Selenium were analyzed.
494

495 ***Main Inclusion and Exclusion Criteria***
496 The main inclusion criteria were participants aged 18-years or above, clinical diagnosis
497 of diuretic-responsive cirrhotic ascites (Child-Pugh score = 7-11 inclusive), abstinence
498 from alcohol for at least 4-weeks prior to screening and written informed consent. The
499 main exclusions were lack of informed consent, use of oral antibiotics,
500 immunosuppressants or antiviral medication within 4-weeks prior to recruitment, change
501 in dose of proton pump inhibitor therapy within 4-weeks before the start of the study
502 treatment, hospital admission for liver-related indication for at least 4-weeks (except
503 paracentesis), BMI > 35 or BMI < 18, presence of a transjugular intrahepatic
504 portosystemic shunt (See protocol in supplementary for details).
505

506 ***Randomization, Dosing and Compliance***
507 Patients were randomized 1:1 to receive 4g of oral Yaq-001 or equivalent placebo nocte
508 for 12-weeks. Treatment compliance was assessed by the number of used or unopened
509 sachets returned to the clinical site at each visit.   Patients taking ≥70% of study
510 medication were considered compliant.
511

512 ***Endpoints and Assessments***
513 <u>Primary Endpoints</u>
514 The main objective of this clinical investigation is to assess the safety and tolerability of
515 Yaq-001 throughout the three months' treatment period.
516

517 <u>Secondary and Exploratory Endpoints</u>

518   Blood and stool samples were collected for later analysis for markers of endotoxemia,
519   systemic inflammation, bile acids, short chain fatty acids, gut permeability and the
520   microbiome (results not reported in this paper).
521
522   **Statistical Analysis**
523   *Animal Studies*
524   Based on the *in vitro* studies, we anticipated a 50% decrease in circulating endotoxin in
525   the treatment groups with an alpha error of 0.05 and power of 80%, resulting in a minimum
526   sample size of 5 animals/group. As this study included several pathophysiological end
527   points, multiple experimental groups were included. All the data accrued from these
528   studies are described in this paper. All the rats in eight groups from three independent
529   batches were included in the analysis as shown in **Fig.S1**. All the mice studied in eight
530   groups were included in **Fig.S2**.
531
532   Group comparisons for continuous variables were performed using Man-Whitney U test
533   (no-normal distribution) or unpaired t-test (normal distribution) and for categorical
534   variables by using Chi-squared test. The data were analyzed using R package (R version
535   4.4.4). 16s microbiome study and circos correlation were analyzed by using Wilcoxon
536   rank sum test and spearman correlation. Software used included Graphpad Prism 9.0
537   (GraphPad software, Inc., San Diego, CA).
538
539   *CARBALIVE-SAFETY Clinical Trial*
540   This first-in-man clinical investigation was not powered to demonstrate statistical
541   significance for any endpoint.   All statistical analyses of study data were carried out using
542   SAS v 9.3 or a later version. For categorical variables, summary tabulations of the number
543   and percentage of patients within each category (with a category for missing data) of the
544   parameter are presented. Percentage calculations are based on non-missing data unless
545   otherwise specified. Please also see statistical analysis plan (Supplementary).
546
547

RESULTS

Functional and Structural Characteristics of Yaq-001

Yaq-001 beads exhibited a consistent pre-defined structure with a bead diameter within the 250-500 μm range and the prescribed porosity (**Fig.S5A**). Yaq-001 rapidly adsorbed albumin (66.5kDa), myoglobin (16.7kDa) and caffeine (0.194kDa) representing different sized biomolecules (**Fig.S5B**). Yaq-001 adsorbed LPS (18kDa) reducing the concentrations from 2.5 to 1.5 EU mL^{-1} (60%) within 30 minutes. No endotoxin was detected in the control solution (0 EU mL^{-1}) (**Fig.S5B**). Yaq-001 also adsorbed a range of bile acids (**Fig.S5C**). Direct co-incubation of Yaq-001 with bacterial suspensions of either *E. coli* or *S. aureus* indicated that Yaq-001 did not affect bacterial growth kinetics for either species following direct contact in comparison to the antibiotic controls (**Fig.S5D**). Mercury porosimetry showed that Yaq-001 used in the clinical trial had a consistent pore size distribution plot in the meso-macroporous range from 30-200 nm (**Fig.S5E**).

Yaq-001 exhibited better performance in adsorptive capacity and effect on endotoxin kinetics than AST-120(**Fig.S5**).

Studies in animal models

Studies in BDL rat model of advanced fibrosis

Effect of Yaq-001 on liver injury and portal pressure

BDL rat model was used to assess the effect of Yaq-001 in cirrhosis (**Fig.1A**). Significant reduction in 4-week body weight was observed in BDL rats (p<0.0001), which was prevented by administration of Yaq-001 (p=0.045) (**Fig.1A**). Yaq-001 was associated with a significantly lower plasma ALT (p=0.007). ALP, TBIL and albumin were not impacted by Yaq-001 (**Fig.S6A, B, C**). Total bile acid concentrations were not different between the BDL and Sham groups and there was no significant impact of Yaq-001 (**Fig.S6E**). MAP was lower in BDL animals and no effect of Yaq-001 was observed (**Fig.S6F**). Yaq-001 resulted in a significant reduction in portal pressure compared to untreated BDL rats [(median (IQR) 11.1 mm Hg (10.3-11.7) *vs* 12.4 mm Hg (10.8-13.3), (p=0.025)] (**Fig.1A**). TUNEL assay showed significantly more intense staining in the liver tissue of BDL compared to Sham rats (**Fig.1A**) (p<0.0001), which was significantly reduced in Yaq-001-

579 treated BDL rats compared to untreated-BDL rats (p=0.025). Collagen proportionate area
580 (CPA) was significantly higher in BDL rats (p=0.0007), which was unchanged with Yaq-
581 001 (p=0.122) (**Fig.S6D**).
582
583 Effect of Yaq-001 on ammonia, organ dysfunction, endotoxemia and bacterial
584 translocation
585 *Ammonia*: Arterial and portal venous ammonia concentrations were significantly
586 increased in BDL rats (p<0.0001), which was significantly reduced by Yaq-001 [(p=0.003)
587 and (p=0.004) respectively] (**Fig.1A**). None of the animals showed signs of overt hepatic
588 encephalopathy.
589
590 *Kidneys:* BDL animals had significantly higher plasma creatinine (p=0.049), which was
591 significantly reduced with Yaq-001 (p=0.025) (**Fig.1A**). Urea was higher in BDL group
592 (p=0.092), which was reduced with Yaq-001 treatment (p=0.095) (**Fig.1A**).
593
594 *Gut permeability, Endotoxemia, Bacterial DNA and Cytokines*: The microbial metabolite,
595 D-lactate, a marker of gut-specific intestinal barrier damage and translocation[16] was
596 significantly increased in BDL rats (p=0.032) and was significantly reduced by Yaq-001
597 (p=0.035) (**Fig.1A**). BDL rats exhibited marked endotoxemia in the portal vein and the
598 artery (p<0.0001 for each), which was significantly reduced with Yaq-001 [(p<0.0001)
599 (p=0.003) respectively] (**Fig.1A**). Portal venous bacterial DNA was detectable in
600 significantly higher number of BDL rats (p<0.05), which was markedly reduced in Yaq-
601 001 administered BDL rats (p=0.08) (**Fig.1A**). Plasma IL-β concentration were higher in
602 the BDL rats but no significant differences were observed in TNF-a, IL-6 and IL-10. No
603 significant changes were seen with Yaq-001 (**Table S1**).
604
605 **Studies in the BDL model of ACLF**
606 This experiment was performed to determine whether Yaq-001 treatment for 2-weeks
607 prevents the occurrence of ACLF when BDL animals are administered LPS (**Fig.S1**,
608 **Fig.1B**).
609

Survival: Animals were sacrificed either at coma stages (considered as a surrogate for mortality) or at 6-hours post LPS. Yaq-001 significantly reduced time to coma of BDL-LPS rats compared to untreated controls (p<0.01) (**Fig.1B**). All animals in the two Sham groups were alive at 6-hours following LPS (data not shown).

Liver: Yaq-001 was associated with significantly lower ALT in BDL-LPS rats compared to untreated rats (p=0.004) (**Fig.1B**). No significant effect of Yaq-001 was observed on ALP, TBIL and albumin (**Fig.S7 A, B, C**). The severity of fibrosis measured using CPA and the body weight were unchanged (**Fig.S7D, E**).

Systemic and Portal hemodynamics: No significant difference in MAP was observed between the groups treated with or without Yaq-001 (**Fig.S7F**) but Yaq-001 produced a significant reduction in portal pressure in BDL-LPS animals compared with the untreated group (p=0.003), (**Fig.1B**).

Brain: Yaq-001 significantly reduced brain water in BDL-LPS compared with the untreated group (p=0.017) (**Fig.1B**). Arterial and portal venous ammonia concentrations were significantly increased in BDL-LPS rats, which was significantly reduced in Yaq-001-treated animals [(p=0.007) and (p=0.017) respectively] (**Fig.1B**).

Kidneys: Creatinine concentrations were significantly higher in BDL-LPS animals (p=0.004), which was significantly reduced by Yaq-001 (p=0.03) (**Fig.1B**).

Cytokines: BDL-LPS group had a significantly higher plasma IL-1β, which was significantly reduced with Yaq-001 (p=0.003). Plasma IL-10 was higher in BDL-LPS and was significantly reduced with Yaq-001 (p=0.028) (**Fig.1B**). No significant differences were observed in IL-6 or TNF-α concentrations between any of the groups (**Table S1**).

Effect of Yaq-001 on peripheral blood cells and Kupffer cells
Significant increase in total leucocyte, neutrophil and monocyte counts in the artery and portal vein were observed with BDL rats (**Fig.S8A, B**) (p=0.008 and p=0.016

respectively), which was significantly reduced with Yaq-001 in the arterial blood and insignificantly reduced in the portal vein (**Fig. S8B**). To determine whether Yaq-001 impacts on the response of peripheral inflammatory cells and Kupffer cells to generate reactive oxygen species (ROS) to LPS *ex vivo,* studies using isolated cells incubated with LPS, were performed. Yaq-001 was associated with significantly lower LPS-induced ROS production in CD163$^-$ Kupffer cells in BDL rats (p=0.036) and portal venous CD43^{hi} monocyte populations of BDL rats (p=0.029) (**Fig.S8C**).

Transcriptomic analysis of gene expression profiles from the Liver, Colon, Brain and Kidneys

Multiorgan transcriptomic analysis was performed to determine the possible molecular mechanisms underlying the clinical effects of Yaq-001. The four groups studied were as follows: Sham (n=3), Sham-Yaq-001 (n=3), BDL (n=3) and BDL-Yaq-001 (n=4) (**Fig.2A**). All differentially expressed genes (DEGs) and related pathways in the liver, colon, kidney and brain are listed in **Table S2**. The top 20 and significant DEGs are listed in **Table S3**.

Effect of Yaq-001 on gene expression profiles in the liver and gut in BDL rats

Liver: Analysis of liver tissue showed 82 DEGs at the threshold of 1.2-fold change and p=0.1 in the four groups (**Fig.2B**). Compared with the Sham group, expression of 62-genes was upregulated, and 15-genes were downregulated in BDL. These significantly changed genes were associated with inflammation, cell death and senescence. Compared to the untreated BDL group, the expression of 7-genes was upregulated and 12-genes were downregulated in the Yaq-001-treated BDL group, indicating the potential role of Yaq-001 in reducing inflammation, cell death and cell senescence. Furthermore, 2-genes were upregulated, and 4-genes downregulated in Sham-Yaq-001 group in comparison to Sham group (**Fig.2C**). Functional analysis demonstrated that BDL rats had enriched pathways related to inflammation, cell senescence, cell death, TLR signaling and other related signaling pathways in comparison with Sham (**Fig.S9A**). Yaq-001 treatment targeted the altered pathways compared with untreated BDL group. Additionally, Yaq-001 treatment also changed the pathways in the liver when compared to Sham group, demonstrating its effect in rats even without cirrhosis (**Fig.S9A**).

25

672

Colon: 43 DEGs were identified from the colonic tissue (**Fig.2D**). 5-genes that correlated with inflammation and cell death were upregulated and 15-genes were downregulated in BDL compared with the Sham group. Moreover, the expression of 10-genes was upregulated, and 13-genes were downregulated with Yaq-001 treatment. Only 1-gene was upregulated in the Sham-Yaq-001 group, and 16-genes were downregulated with Yaq-001 compared with the untreated Sham group (**Fig.2E**). Functional analysis indicated that inflammation, cell senescence, cell death, TLR signaling and intracellular signaling were associated with BDL in comparison with the Sham group (**Fig.S9B**). Yaq-001 targeted the altered pathways, indicating the potential mechanisms in the prevention of gut dysfunction and permeability (**Fig.S9B**).

Effect of Yaq-001 on gene expression profiles in the brain and kidney in BDL rats

Brain: 17 DEGs were identified from the brain tissue (**Fig.2F**). Compared with Sham group, expression of 2-genes was upregulated and 13-genes were downregulated in BDL animals. These significantly changed genes were associated with inflammation, cell death, and cell senescence. Compared to the untreated-BDL group, the expression of 5-genes was upregulated and 2-genes were downregulated in the Yaq-001-treated BDL group (**Fig.2G**). Functional analysis demonstrated that BDL rats had enriched pathways related to inflammation, cell senescence, cell death, TLR signaling and intracellular signaling (**Fig.S9C**). Yaq-001 targeted cytokine-cytokine receptor interaction, cytosolic DNA-sensing pathway, TLR signaling pathway, NOD-like receptor signaling pathway, neutrophil extracellular trap formation, TGF-beta signaling pathway and cytokine-cytokine receptor interaction pathways compared to untreated-BDL group (**Fig.S9C**).

Kidneys: 30 DEGs were identified from kidney tissue (**Fig.2H**). 9-genes that correlated with inflammation were downregulated in BDL. The expression of 5-genes was upregulated and 4-genes were downregulated with Yaq-001 treatment compared to untreated-BDL group. 5-genes were upregulated in Sham-Yaq-001 group, and 3-genes were downregulated with Yaq-001 compared with untreated-Sham group (**Fig.2I**). Functional analysis indicated that inflammation and TLR signaling were associated with

703 BDL in comparison with Sham (**Fig.S9D**). Compared with the untreated-BDL group, Yaq-
704 001 targeted the altered pathways, indicating the potential mechanisms in the prevention
705 of renal dysfunction (**Fig.S9D**).
706

707 ***Effect of Yaq-001 on the gut microbiome profile***
708 The effects of Yaq-001 on the microbiome bacterial composition was assessed by
709 metataxonomics. At the family level, an abundance of six bacteria were significantly
710 changed at the threshold of 2-fold change and *Porphyomonadaceae* was significantly
711 changed ($p < 0.05$) comparing BDL with Sham **(Fig.3A)**. At genus level, 19 bacteria
712 including were significantly changed in abundance. *Barnesiella* was significantly changed
713 ($p < 0.05$) comparing BDL with Sham group **(Fig.3B)**. These changes were reversed with
714 Yaq-001 treatment compared to untreated-BDL rats (**Fig.S10A, B**, **Table S4 and**
715 **Fig.S10C, D).** For between groups sample diversity, PERMANOVA analysis revealed a
716 significant difference in beta diversity between groups ($R^2 = 0.32$, $p = 0.001$). Yaq-001
717 appeared to moderately restore the beta diversity in the BDL group especially in PCoA2
718 axis (**Fig.S10E, F**).
719

720 To further investigate the potential importance of the changes in the microbiome induced
721 by Yaq-001, we correlated these with all significantly changed DEGs and the top 20 DEGs
722 in the four organs. Circos plots indicated a significant correlation between them (**Fig.3C,**
723 **D and Fig.S11A, B**, **C)**. *Porphyromonadaceae*, was observed to positively correlate with
724 three DEGs - TGFB2 and CASP1 in liver tissue, and FOS in colonic tissue. Also, it
725 correlated negatively with five DEGs- TGFB2, IL-18 and CCR5 in brain tissue, CXCL10
726 in colon tissue and CCL24 in kidney tissue.
727

728 ***Effect of Yaq-001 on metabolomic profile***
729 Significant difference of acetate/creatinine, glycine/creatinine, lactate/creatinine,
730 betaine/creatinine, trimethylamine oxide/creatinine and bile acid/creatinine ratio were
731 observed in BDL compared to Sham. Treatment of BDL rats with Yaq-001 resulted in
732 significant resolution of acetate/creatinine, glycine/creatinine and lactate/creatinine
733 compared to the untreated BDL animals (**Fig.S12**).

734

Studies in CCl₄ mice

Effect of Yaq-001 on liver injury and fibrosis

6-week and 12-week CCl₄ mice models were used to further confirm the effect of Yaq-001 in cirrhosis (**Fig.4A，B**). Yaq-001 was associated with a significantly lower plasma ALT ($p<0.0001$, $p<0.0001$) in both 6-week and 12-week CCl₄ models. ALP and TBIL were reduced by Yaq-001 in 6-week CCl₄ mice ($p=0.040$, $p=0.001$) (**Fig.4A，B**). CPA was significantly higher in both CCl₄ mice compared with control animals ($p=0.0001$, $p=0.0001$), which was significantly reduced with Yaq-001 ($p=0.024$, $p=0.012$) (**Fig.4A, B**). TUNEL assay showed significantly more intense staining in the liver tissue of CCl₄ compared to Control mice ($p<0.001$, $p<0.001$), which was significantly reduced in Yaq-001-treated CCl₄ mice compared to untreated-CCl₄ mice ($p=0.021$, $p=0.017$) (**Fig.4A, B**).

Effect of Yaq-001 on ammonia, organ dysfunction and endotoxemia

Ammonia: Ammonia concentrations were significantly increased in the 6-week and 12-week CCl₄ mice compared with controls ($p=0.002$, $p=0.001$), which was significantly reduced by Yaq-001($p=0.025$, $p=0.035$) (**Fig.4A, B**). None of the animals showed signs of hepatic encephalopathy.

Kidneys: Higher plasma creatinine was significantly reduced by Yaq-001 treatment ($p=0.005$, $p=0.003$) in 6-week and 12-week CCl₄ animals (**Fig.4A, B**).

Endotoxemia: Both 6-week and 12-week CCl₄ mice exhibited marked endotoxemia compared with controls ($p=0.007$, $p=0.007$), which was significantly reduced with Yaq-001($p=0.007$, $p=0.043$) (**Fig.4A, B**).

In vitro **studies in intestinal organoids to assess gut permeability.**

Intestinal organoids were successfully derived and cultured from small intestine of C57BL/6 mice. Intestinal organoids underwent eversion into apical-out polarity in the first 12h of suspension culture and collected for identification and subsequent experiments (**Fig.5A**). Immunostaining of the microvilli (mv; F-actin) demonstrated that intestinal

organoids in suspension had reversed polarity such that the apical surface faces outward (**Fig.5A**). Apical-out intestinal organoids possessed goblet cells, which were identified with MUC2 staining (**Fig.5B**). Gut permeability of apical-out intestinal organoids was significantly increased by coculturing with fecal water from CCl_4 group compared with the control group (p=0.003) (**Fig.5C,D**). The gut permeability was significantly decreased with fecal water from Yaq-001 treated CCl_4 animals compared with the CCl_4 group (p=0.001) (**Fig.5C,D**).

CARBALIVE-SAFETY Clinical Trial

The data regarding safety and tolerability are reported here. Other secondary and exploratory end points will be described elsewhere.

Patient Characteristics

Thirty-four-patients were screened for this study at 8-European centers. 28-patients met the study entry criteria and were randomized to either active or placebo groups. Six-patients screened did not meet the study entry criteria. Dosing was not initiated in 2-patients randomized to placebo due to withdrawal of consent (**Fig.S3**, CONSORT). 3-patients were included for the second dosing cohort of 8 g. This part of the study was terminated prematurely due to the coronavirus pandemic with none of the patients completing the study duration (data not included).

In accordance with study entry criteria, all patients had cirrhosis with diuretic-responsive ascites and Child-Pugh score of 7-8. The baseline demographics were similar across treatment groups. The ratio of male patients to female patients was reflective of the disease state. Compliance in the active and placebo groups was 92.9% and 66.7% respectively (**Table 1**).

Safety and Tolerability

Of the 14-patients enrolled in the Yaq-001 treatment group, 13 (93%) completed 12-weeks of therapy. The median duration of exposure was 83 (6-94) days. Ten of the 12 (83%) patients who received placebo completed the treatment. The median duration of

796　exposure was 83 (14-86) days. No deaths or serious adverse events were reported in the
797　study. The difference in treatment-emergent adverse events (TEAEs) in patients treated
798　with Yaq-001 and those treated with placebo is presented in **Table 2**. The most frequently
799　reported TEAEs were gastrointestinal in nature in both the active and placebo groups. Of
800　these, only constipation and diarrhea were evaluated by the clinical investigator as
801　possibly related to the investigational product. One placebo-treated patient withdrew from
802　the study due to diarrhea.

803

804　Across both treatment groups, 40/51(78%) of the reported TEAEs were evaluated by the
805　clinical investigator as not related or unlikely related (32/38; 84% for the active treatment
806　group; 8/13; 62% for the placebo group). The incidence of adverse events reported is
807　reflective of the targeted subject population for this clinical investigation. The majority of
808　the TEAEs reported were not considered by the clinical investigator to be related to
809　treatment and were mild in intensity. Systemic antibiotics were administrated for the
810　following TEAEs in the active arm: Amoxicillin – acute bronchitis; Clarithromycin – acute
811　bronchitis; Phosphomycin – urinary tract infection. None of these infections were related
812　to the administration of the investigational product. Drugs received by the patients at the
813　time of randomization and during follow up are listed in Supplementary Tables.
814　Treatment-emergent, clinically significant laboratory abnormalities are listed in **Table 3**.
815　None were deemed treatment-related by the investigator.

816

817　***Clinical, hematological and biochemical variables***
818　The data are summarized in **Table 3**. No significant changes in any of the clinical
819　parameters were observed in any of the groups. Although there was a trend towards a
820　reduction in the white cell count and C-reactive protein in the Yaq-001 group, the
821　differences were not significant.

822

823　***Nutritional status***
824　No significant differences were observed in either treatment group with regards to global
825　nutritional status, vitamin B12 and folate, Vitamin A or E, or copper zinc, and selenium.
826　Median vitamin A, zinc and baseline vitamin D concentrations were below the limit of

827 normal range but no differences between treatment groups were observed. No changes
828 were observed in any of the micronutrient parameters with treatment and these were
829 evenly matched between groups. Any baseline abnormalities were attributable to the
830 underlying natural history of cirrhosis.
831
832
833

Discussion

The results of the study showed that Yaq-001 prevented progression of liver injury and fibrosis in animal models of cirrhosis and significantly reduced the mortality of ACLF animals. This was associated with positive impact on markers of gut permeability, liver injury, portal pressure, brain and kidneys. These pleiotropic effects of Yaq-001 were associated with partial restoration of the composition of the microbiome bacterial community, reduction in the severity of endotoxemia, ammonia, inflammation, cell death, signaling pathways and LPS sensitivity. A Phase 2 equivalent, double-blind, multicenter, placebo-controlled, randomized clinical trial in patients with cirrhosis confirmed regulatory compliance and, safety and tolerability of Yaq-001, thereby, providing evidence of clinical translatability. The data provide the rationale to proceed to further clinical trials.

Translocation of bacteria, its products and metabolites are critically important in the progression of hepatic fibrosis and pathogenesis of complications of cirrhosis[1,17-20]. Indeed, selective gut decontamination using norfloxacin or rifaximin are the current standard of care for secondary prophylaxis of patients with spontaneous bacterial peritonitis and hepatic encephalopathy respectively[21-22]. However, the use of these antibiotic strategies is limited to patients with advanced cirrhosis and induces the risk of antibiotic resistance.[23] The data presented here provide a safe, gut-restricted, non-antibiotic strategy, Yaq-001, which has the potential to diminish translocation and prevent the progression of hepatic injury, fibrosis and, prevent extrahepatic organ injury in models of cirrhosis. The *in vitro* studies demonstrate that Yaq-001 has the optimal pore size distribution to bind intraluminal factors such as free endotoxin. We also tested *in vitro* bacterial growth kinetics of two species, which were not affected by Yaq-001, an observation that was subsequently confirmed with studies in the BDL animal model where no change diversity were observed.

Endotoxemia has also been implicated in immune dysfunction resulting in a dysregulated systemic inflammatory response, which is strongly associated with the progression of fibrosis, cirrhosis and occurrence of ACLF[24,25]. Yaq-001 reduced the severity of endotoxemia and bacterial DNA positivity, which was associated with attenuated systemic

32

inflammation. Significant improvements in LPS-induced ROS production were observed in trafficking portal venous monocytes suggesting that Yaq-001 had attenuated the primed state of monocyte/macrophage populations within the gut-liver axis. This observed reduction in LPS-induced ROS production may be important in explaining the reduction in plasma IL-1β in LPS-treated BDL rats.

Plasma D-lactate, a marker of increased gut permeability was reduced in the Yaq-001 treated BDL rats[26]. Elevated plasma D-lactate levels in cirrhosis is associated with decompensation[22].   Transcriptomic analysis of colonic tissue demonstrated upregulation of genes associated with necroptosis, apoptosis and inflammation in BDL animals. Functional analyses pointed to modulation of colonic inflammation by Yaq-001, IL-17 signaling, which is known to have diverse biological functions, promoting protective immunity against many pathogens, neutrophil recruitment, antimicrobial peptide production and enhanced barrier function[27, 28]. To further validate the potential effect of Yaq-001 in modulating gut permeability, we performed experiments in intestinal organoids that were incubated with fecal water[29]. The data confirmed that even in *in vitro* settings, fecal water obtained from the feces of CCL4-induced cirrhosis animals enhanced permeability of the organoids, which was prevented in the fecal water obtained from animals that were treated with Yaq-001. The data support the hypothesis that Yaq-001 impacts on the factors in the gut responsible for increasing gut permeability in cirrhosis.

Yaq-001 significantly reduced the severity of liver injury and portal hypertension in both the BDL and BDL-LPS models of cirrhosis and ACLF. The lack of significant differences in CPA between untreated and Yaq-001-treated BDL groups suggests that the reduction in portal pressure is possibly due to modulation of the dynamic component of portal hypertension, in which inflammation is known to play a role[30,31] and proposes Yaq-001 as a potential treatment for portal hypertension. Reduction in ALT levels and TUNEL staining confirmed a reduction in liver injury in the Yaq-001 treated animals. The reduction in liver injury in the LPS treated BDL animals suggests that Yaq-001 has a particular effect on endotoxin sensitivity *in vivo.*   This hypothesis was tested in isolated Kupffer cells, which

895   confirmed that LPS-induced ROS production was significantly impacted by Yaq-001
896   treatment.

897

898   Transcriptomic analysis of liver tissue demonstrated that the upregulated genes,
899   CXCL16, CASP1 and TGFB2 in BDL rats was prevented by Yaq-001 administration.
900   Silencing of CXCL16 alleviates hepatic ischemia reperfusion injury and CXCL16 variant
901   is also associated with Hepatitis B virus related acute liver failure[32]. CASP1 mediates pro-
902   inflammatory cytokine release and pyroptotic cell death in cirrhosis and its inhibition has
903   been shown to prevent ACLF [33]. TGFB2 is an important mediator of cellular senescence[34,
904   35]. Of note, Yaq-001 also modified necroptosis and cytosolic DNA-sensing pathways
905   representing cell death, which are known to be activated by LPS that can trigger systemic
906   inflammation[36]. These effects of Yaq-001 potentially explains the effect of Yaq-001 in
907   reducing liver injury [37, 38].

908

909   Yaq-001 administration had a significant impact on time to coma of ACLF rats, which is
910   considered as a surrogate for mortality compared to untreated controls. Yaq-001 also
911   significantly lowered portal venous and arterial ammonia levels, which was associated
912   with reduced brain water. Transcriptomic analysis of brain tissue showed that IL-18,
913   TGFB2, CCR5 and IL-23A were dysregulated in BDL rats and these were corrected by
914   Yaq-001. IL-18 is released during pyroptosis by activation of the inflammasome complex
915   in neuroinflammatory and neurodegenerative diseases[39]. The effect of Yaq-001 on
916   TGFB2 may mean that it has an impact on senescence, which is known to be associated
917   with hepatic encephalopathy. CCR5 has been implicated in neuroprotection and is novel
918   therapeutic target in stroke[40]. The impact of Yaq-001 on IL-23A indicates possible
919   reduction in neuroinflammation.

920

921   In both cirrhosis and ACLF models, Yaq-001 reduced renal dysfunction. Transcriptomic
922   analysis of kidney tissue showed that CCL24 was downregulated in BDL rats, which was
923   prevented in the Yaq-001-treated animals. CCL24 protects renal function in the
924   development of early diabetic nephropathy by exerting an anti-inflammatory effect[41]. Yaq-

925 001 impacted, on the cytokine-cytokine receptor interactions and chemokine and toll-like
926 signaling pathways, which were abnormal in the BDL rats.

928 BDL animals become sarcopenic and lose weight[42], which was significantly abrogated by
929 Yaq-001. The possible mechanisms underlying this effect are likely multifactorial[43]. Yaq-
930 001 reduced ammonia significantly, which has been shown to induce sarcopenia[44].
931 Weight loss in cirrhosis is also attributed to an increased catabolic state in the context of
932 systemic inflammatory response and thus the observed improvement in body weight may
933 reflect the diminished catabolic state with reduced inflammation[43].

935 The clinical effects of Yaq-001 observed in the BDL models was validated in the CCL4
936 induced liver injury animal models. Two models were studied. In the first (6-week model),
937 Yaq-001 was administered in a preventative mode starting its administration with the
938 onset of liver injury during parenteral administration of CCL4. The results showed
939 significant reduction in the severity of liver injury, fibrosis and progression to cirrhosis,
940 endotoxemia, creatinine and ammonia levels. In the second (12-week model), Yaq-001
941 was administered starting at 6-weeks when the animal already had advanced fibrosis/
942 cirrhosis. Again, significant reduction in markers of liver injury, fibrosis, endotoxemia,
943 creatinine and ammonia were observed. Extrapolating these observations to the humans,
944 the results from the 6-week model suggests that Yaq-001 may well be useful to prevent
945 the progression of fibrosis in patients without cirrhosis and, from the 12-week model, the
946 possibility of prevention of progression of liver disease in those with well compensated
947 cirrhosis.

949 Gut microbiota are important in modulating intestinal health, permeability, bacterial
950 translocation, systemic inflammation and complications of cirrhosis[45-47]. BDL was
951 associated with marked changes in the abundance of microbiota, which were reversed
952 by Yaq-001. In particular, the abundance of *Porphyromonadaceae* and *Barnesiella* were
953 significantly elevated in BDL rats and significantly decreased with Yaq-001. This change
954 is potentially important as *Porphyromonadaceae* is a pro-inflammatory bacterium that has
955 been positively correlated with hepatic encephalopathy[48] and, *Barnesiella* and

956 *Porphyromonadaceae* have been associated with liver cancer [49,50]. Urinary NMR analysis
957 reflects the combined metabolic status of both host and microbiota. Yaq-001 was
958 associated with a distinct shift of acetate, glycine and lactate in metabolomic profile in
959 BDL rats. These metabolites are generated by mixed acid fermentation (MAF), typically
960 by bacteria such as Enterobacter. MAF is not the preferred metabolic pathway for
961 facultative anaerobes and may be indicative that Enterobacter populations are under
962 conditions of metabolic stress in Yaq-001 treated BDL animals. As these species are
963 often pathogenic in cirrhosis, this may represent a beneficial change. However, the exact
964 mechanisms by which the change in the microbiome results in improvement in distant
965 organ function and gene expression cannot be directly inferred from the data derived from
966 this study. One possibility is that alongside LPS adsorption and modulation of other
967 unmeasured toxins, the *milieu* of the gut is changed allowing proliferation of more
968 autochthonous bacteria[47], which impacts on gut inflammation that reduces gut
969 permeability. This hypothesis is supported by the organoid experiments. Reduction in
970 permeability would result in a reduction in endotoxemia, systemic inflammation,
971 improvement of organ function and LPS-sensitivity. In this study, most of these changes
972 have been described individually but whether this is happening in sequence has not been
973 investigated.

974

975 As Yaq-001 is completely excreted unchanged in the stool, it is regulated in Europe as a
976 device but as a drug in the US, the clinical trial was performed both according to ISO
977 standards and ICH-GCP guidance. The results of this first-in-man randomized, placebo-
978 controlled trial suggested that oral Yaq-001 at a dose of 4g nocte was well tolerated with
979 a favorable safety profile. Despite the rapid adsorption kinetics for bacterial toxins and
980 metabolites, Yaq-001 treatment had no negative impact on micronutrient levels or impact
981 on nutritional profile as assessed by the gold standard Royal Free Global Assessment
982 tool. This data must be interpreted keeping in mind that Yaq-001 was administered post
983 prandially, separated from meals by 4-hours and from drugs by about 6-hours as
984 necessitated by the protocol. It is important to note that the studies were performed in
985 stable cirrhosis patients, many of whom had minimal evidence of systemic inflammation
986 and therefore, any clinical effect of this intervention was difficult to gauge. However, future

987 analysis of the available samples from the blood and stool will provide answers as to
988 whether Yaq-001 modulates the gut microbiome, inflammation and endotoxemia.

989

990 These results must be considered in view of some limitations. First, the rodent
991 microbiome is not directly analogous to the human and further clinical studies will be
992 required to verify the effects on the gut microbiome's bacterial composition. Second,
993 although Yaq-001 was effective in adsorbing a variety of bile acids *in vitro* and reduced
994 bile acids significantly in Sham animals, no impact on bile acids was seen in BDL animals.
995 This possibly reflects the effect of the BDL model, where no increase in bile acids was
996 observed. Also, no changes in bile acids were observed in CCL4 animals but these
997 animals did not have elevated bile acid either. Third, although, Yaq-001 was observed to
998 impact positively on the gene expression profiles of multiple pathways, their exact
999 relevance at the protein or cellular level has not been explored. Fourth, as only one dose
1000 of Yaq-001 was tested in the clinical trial, further dose-ranging studies will be needed to
1001 define optimal dosing for safety and efficacy. However, the animal toxicity studies that
1002 were performed by an independent laboratory for regulatory purposes, showed evidence
1003 of safety in much larger doses than that administered in the present studies (summary in
1004 Supplementary).

1005

1006 In conclusion, the data provides compelling evidence for the potential of Yaq-001 as a
1007 novel therapy targeting the gut microbiome, bacterial translocation and gut permeability
1008 that impacts on systemic inflammation, liver injury and fibrosis and, organ function in
1009 models of cirrhosis and improves survival in ACLF. The placebo-controlled clinical trial of
1010 Yaq-001 in cirrhosis patients provides evidence of safety and tolerability allowing
1011 translation to next phase of clinical studies to define its potential as a novel therapeutic
1012 for patients with cirrhosis.

1013

Abbreviations

ACLF, acute-on-chronic liver failure; LPS, lipopolysaccharides; BDL, bile duct ligation; ALT, alanine aminotransferase; ALP, alkaline phosphatase; TBIL, total bilirubin; MAP, mean arterial pressure; CPA, collagen proportionate area; PSR, picrosirus red ; PP, portal pressure; ROS, reactive oxidant species; DEGs, differential expressed genes; KEGG, Kyoto Encyclopedia of Genes and Genomes; TLR, toll-like receptor; TNF-a, tumor necrosis factor-a.

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Figure legends

Fig.1. Effect of Yaq-001 on organ dysfunction, endotoxemia and bacterial
translocation in BDL and ACLF rats.

1179 (A) Rats underwent bile duct ligation for 4-weeks as a model of cirrhosis (n=23-37/group).
1180 Treatment groups received Yaq-001 for 2 weeks before sacrifice.

1181 *4-week body weight in four groups*: Sham (n=31), Sham-Yaq-001 (n=24), BDL (n=31)
1182 and BDL-Yaq-001 (n=38). Significantly lower final body weights were observed in BDL
1183 compared to Sham controls (p<0.001). Yaq-001-treated BDL rats had a significantly
1184 higher body weights compared to untreated-BDL rats (p<0.05).

1185 *Plasma alanine transaminase (ALT) concentrations* in Sham (n=17), Sham-Yaq-001
1186 (n=14), BDL (n=17) and BDL-Yaq-001 (n=26) groups and *Portal pressure (PP)*
1187 *measurements in Sham (n=17), Sham-Yaq-001 (n=19), BDL (n=14) and BDL-Yaq-001*
1188 *(n=26) groups*. Significantly higher ALT and PP were observed in BDL compared to Sham
1189 controls (p<0.0001). Yaq-001-treated BDL rats had a significantly lower ALT and PP
1190 compared to untreated-BDL rats (p<0.01, p<0.05).

1191 *TUNEL assay of liver tissue with quantification of staining by digital image analysis*.
1192 Significantly higher TUNEL staining was observed in BDL compared to Sham controls
1193 (p<0.0001). Yaq-001-treated BDL rats had a significantly lower TUNEL staining
1194 compared to untreated-BDL rats (p<0.05) indicative of a reduction in liver cell death with
1195 Yaq-001 treatment.

1196 *Arterial ammonia concentrations i*n Sham (n=7), Sham-Yaq-001 (n=5), BDL (n=19), BDL-
1197 Yaq-001 (n=21) groups and *Portal venous ammonia concentrations* in Sham (n=6),
1198 Sham-Yaq-001 (n=5), BDL (n=13), BDL-Yaq-001 (n=18) groups. Significantly increased
1199 arterial ammonia concentrations and portal venous ammonia concentrations were
1200 observed in BDL compared to Sham controls (p<0.0001, p=0.0001). Yaq-001 significantly
1201 decreased arterial and portal venous ammonia concentrations in BDL rats (p<0.01 for
1202 both).

1203 *Serum creatinine* in Sham (n=19), Sham-Yaq-001 (n=17), BDL (n=20), BDL-Yaq-001
1204 (n=17) and *serum urea* in Sham (n=28), Sham-Yaq-001 (n=23), BDL (n=30), BDL-Yaq-
1205 001 (n=34) groups. Yaq-001 markedly decreased serum creatinine levels in BDL rats
1206 (p<0.05).

Plasma D-lactate in Sham (n=7), Sham-Yaq-001 (n=8), BDL (n=6), BDL-Yaq-001 (n=7). Plasma D-lactate was significantly increased in the BDL group compared with Sham animals (p<0.05). Yaq-001 resulted in a significant reduction in plasma D-lactate in BDL rats (p<0.05).

Portal venous endotoxin [Sham (n=6), Sham-Yaq-001 (n=5), BDL (n=12) and BDL-Yaq-001 (n=7)] and *arterial endotoxin* [Sham (n=6), Sham-Yaq-001 (n=5), BDL (n=12) and BDL-Yaq-001 (n=7)].

Portal venous bacterial DNA positivity [Sham (n=6), Sham-Yaq-001 (n=5), BDL (n=12) and BDL-Yaq-001 (n=13)] and *arterial plasma bacterial DNA positivity* [Sham (n=6), Sham-Yaq-001 (n=6), BDL (n=12) and BDL-Yaq-001 (n=7)]. Significantly higher portal venous endotoxin and arterial endotoxin were observed in BDL rats compared to Sham rats (p<0.0001). Significantly higher portal venous plasma bacterial DNA positivity was observed in BDL rats compared to Sham rats (p<0.05). Yaq-001 administration was associated with a significant reduction of portal venous and arterial endotoxin compared to untreated-BDL rats (p<0.0001, p<0.01). Yaq-001 administration reduced bacterial DNA positivity, which was not statistically different (p>0.05).

(B) Rats underwent sham biliary surgery or BDL for 4-weeks. The treated group received Yaq-001 for 2 weeks prior to LPS injection. Animals were sacrificed either at coma stages or 6 hours after LPS injection (n=9-16/group).

Kaplan-Meier analysis of BDL-LPS rats with (n=16) or without (n=12) Yaq-001 treatment. Yaq-001 treatment significantly improved the survival of BDL-LPS rats compared to untreated-BDL-LPS rats (log rank test, p=0.003).

Plasma ALT concentrations in Sham-LPS (n=7), Sham-LPS-Yaq-001 (n=5), BDL-LPS (n=10) and BDL-LPS-Yaq-001 (n=9) groups and *PP measurements* in Sham-LPS (n=8), Sham-LPS-Yaq-001 (n=10), BDL-LPS (n=9) and BDL-LPS-Yaq-001 (n=9) groups. Yaq-001-treated BDL-LPS rats had a significantly lower ALT and PP compared to untreated-BDL-LPS rats (p<0.005).

Brain water percentage in Sham-LPS (n=4), Sham-LPS-Yaq-001 (n=4), BDL-LPS (n=7), BDL-LPS-Yaq-001 (n=13) groups. *Arterial ammonia* concentrations in Sham-LPS (n=5), Sham-LPS-Yaq-001 (n=5), BDL-LPS (n=7), BDL-LPS-Yaq-001 (n=7) groups. *Portal*

venous ammonia concentrations in Sham-LPS (n=5), Sham-LPS-Yaq-001 (n=5), BDL-LPS (n=6), BDL-LPS-Yaq-001 (n=5) groups. Yaq-001 decreased brain water percentage and arterial/portal venous ammonia concentrations in BDL-LPS rats compared to untreated rats ($p < 0.05$, $p < 0.01$, $p < 0.05$).

Serum creatinine in Sham-LPS (n=4), Sham-LPS-Yaq-001 (n=3), BDL-LPS (n=12) and BDL-LPS-Yaq-001 (n=6) groups. Serum urea in Sham-LPS (n=8), Sham-LPS-Yaq-001 (n=4), BDL-LPS (n=12) and BDL-LPS-Yaq-001 (n=8) groups. Yaq-001 significantly decreased creatinine levels in BDL-LPS rats ($p < 0.05$).

Plasma cytokines in Sham-LPS (n=6), Sham-LPS-Yaq-001 (n=9), BDL-LPS (n=8) and BDL-LPS-Yaq-001 (n=8) groups. Yaq-001 significantly decreased plasma IL-1β and IL-10 concentrations in BDL-LPS groups ($p < 0.01$, $p < 0.05$).

Fig.2.Effect of Yaq-001 on gene expression profiles in the multiorgans in BDL rats. (A) Rats underwent BDL for 4-weeks as a model of cirrhosis (n=3-4/group) and the treatment groups received Yaq-001 for 2-weeks before sacrifice. Liver, colon, brain and kidney were collected for transcriptomic analysis. (B, D, F, H) Heatmap of differentially expressed genes (DEGs) in different organs between Sham (n=3), Sham-Yaq-001 (n=3), BDL (n=3) and BDL-Yaq-001 (n=4) groups. DEGs were identified at 1.2-fold change and p=0.1 threshold in three pairwise groups (BDL versus Sham, BDL-Yaq-001 versus BDL, Sham-Yaq-001 versus Sham). (C, E, G, I) Volcano plot of pairwise DEGs in four organs among Sham (n=3), Sham-Yaq-001 (n=3), BDL (n=3) and BDL-Yaq-001 (n=4) groups. The vertical dashed lines indicated the threshold for 1.2-fold change. The horizontal dashed line indicated the adjusted p=0.05 and p=0.1 threshold. The right part indicates up-regulation of gene expression, and the left part indicates down-regulation of gene expression. The top 20 genes are indicated by gene names.

Fig.3. Effect of Yaq-001 treatment on the microbiome composition. (A) Heatmap of gut microbiome associated with the effect of Yaq-001 as determined by 16S PCR at the family level. The Family *Porphyromonadaveae* with asterisk was statistically differently abundant between BDL (n=7) vs Sham (n=6), and between BDL-Yaq-001 (n=7) vs BDL groups (n=7) (Wilcoxon rank sum test, $p < 0.05$). The abundance of this family was

statistically higher in BDL group than in Sham group, and its abundance statistically decreased in the BDL-Yaq-001 group than in the BDL group. The other six families in the heatmap were with marked fold changes between BDL vs Sham, and between BDL-Yaq-001 vs BDL groups (|log2FC|>2). Of these, five were more abundant in the BDL group than in the Sham group. The abundance largely decreased in the Yaq-001-treated group. In addition, of these, one family was less abundant in the BDL group than in the Sham group. The abundance increased in the Yaq-001-treated group. (B) Heatmap of gut microbiome at the Genus level. The Genus *Barnesiella* with asterisk was statistically differently abundant between BDL vs Sham, and between BDL-Yaq-001 vs BDL groups (Wilcoxon rank sum test, p<0.05). The abundance of this genus was statistically higher in BDL group than in the Sham group, and its abundance statistically decreased in the BDL-Yaq-001 group. The other 19 genera in the heatmap represent those with significant fold change values between BDL vs Sham, and between BDL-Yaq-001 vs BDL groups (|log2FC|>2). Of these, 14 were more abundant in the BDL group compared with the Sham group. The abundance decreased in the Yaq-001-reated BDL group. In addition, 5 genera were less abundant in the BDL group than in the Sham group. Their abundance increased in the Yaq-001-treated BDL animals. (C, D) Correlation plots between markedly changed genes and gut microbiome at family/genus. The genes were from amongst the top 20 changed genes in BDL animals with Yaq-001 treatment. Nodes represent either genes (lower semi-circular part) or bacteria (upper semi-circular part) at the family and genus level. The nodes are colored based on the log-fold change for the differential gene expression and differences in the bacterial abundance. The red nodes indicate an increase and blue nodes indicate a decrease. Edges represent the correlation coefficient calculated between genes and microbial genus or family with red indicating a positive correlation and blue a negative correlation. Correlation coefficients greater or equal to 0.4 were plotted in plot C (Spearman's coefficient >= 0.4), and D shows all correlations.

Fig.4. Effect of Yaq-001 on organ dysfunction, ammonia and endotoxemia in CCl$_4$ mice.

(A) Mice underwent CCl$_4$ injection for 6-weeks as a model of cirrhosis (n=6-12/group) and the treatment groups received Yaq-001 for 6 weeks before sacrifice.

Plasma ALT, ALP and TBIL concentrations in Control (n=6), Control-Yaq-001 (n=6), 6-week CCl_4 (n=12) and 6-week CCl_4-Yaq-001 (n=12) groups. Significantly higher ALT, ALP and TBIL were observed in CCl_4 compared to controls (p=0.0001, p=0.0007, p=0.012). Yaq-001-treated CCl_4 mice had a significantly lower ALT, ALP and TBIL compared to untreated-CCl_4 mice (p<0.0001, p=0.040, p=0.001).

H&E and PSR staining of liver tissue. CCl_4 mice were associated with a significant increase in CPA compared to Controls (p=0.0001). Yaq-001 had significant effect on CPA in CCl_4-Yaq-001 compared to CCl_4 mice(p=0.024).

TUNEL staining liver tissues. Significantly greater staining was observed in CCl_4 compared to Controls (p=0.0001). Yaq-001-treated CCl_4 mice had a significantly lower TUNEL staining compared to untreated-CCl_4 (p=0.021) with Yaq-001 treatment.

Venous ammonia concentrations and serum creatinine levels in Control (n=6), Control-Yaq-001 (n=6), 6-week CCl_4 (n=12) and 6-week CCl_4-Yaq-001 groups(n=12). Significantly increased ammonia concentrations were observed in CCl_4 compared to Controls (p=0.0020). Yaq-001 significantly decreased venous ammonia concentrations and serum creatinine levels in CCl_4 mice (p=0.025, p=0.005).

Venous endotoxin concentrations in Control (n=3), Control-Yaq-001 (n=3), 6-week CCl_4 (n=10) and 6-week CCl_4-Yaq-001 groups(n=10). Significantly higher venous endotoxin was observed in CCl_4 mice compared to Control mice (p=0.007). Yaq-001 administration was associated with a significant reduction of venous endotoxin compared to untreated-CCl_4 mice (p=0.007).

(B) Mice underwent CCl_4 injection for 12-weeks as a model of cirrhosis (n=6-12/group) and the treatment groups received Yaq-001 for 6-weeks before sacrifice.

Plasma ALT, ALP and TBIL concentrations in Control (n=6), Control-Yaq-001 (n=6), 12-week CCl_4 (n=12) and 12-week CCl_4-Yaq-001 (n=12) groups. Significantly higher ALT, ALP and TBIL were observed in CCl_4 compared to controls (p=0.0001, p=0.0008, p=0.007). Yaq-001-treated CCl_4 mice had a significantly lower ALT compared to untreated-CCl_4 mice (p<0.0001).

1329 *H&E and PSR staining of liver tissue* in CCl$_4$ mice. CCl$_4$ mice were associated with a
1330 significant increase in CPA compared to Controls (p=0.0001). Yaq-001 had significant
1331 effect on CPA in CCl$_4$-Yaq-001 compared to CCl$_4$ mice(p=0.012).
1332 *TUNEL staining of liver tissues*. Significantly higher TUNEL staining was observed in CCl$_4$
1333 compared to Controls (p=0.0001). Yaq-001-treated CCl$_4$ mice had a significantly lower
1334 TUNEL staining compared to untreated-CCl$_4$ (p=0.017) indicative of a reduction in liver
1335 cell death with Yaq-001 treatment.
1336 *Venous ammonia*. Significantly increased ammonia concentrations were observed in
1337 CCl$_4$ compared to Controls (p=0.001). Yaq-001 significantly decreased venous ammonia
1338 concentrations in CCl$_4$ mice (p=0.035).
1339 *Serum creatinine*: Yaq-001 significantly decreased serum creatinine levels in CCl$_4$ mice
1340 (p=0.003).
1341 *Venous endotoxin concentrations* in Control (n=3), Control-Yaq-001 (n=3), 12-week CCl$_4$
1342 (n=10) and 12-week CCl$_4$-Yaq-001 groups(n=10). Significantly higher venous endotoxin
1343 was observed in CCl$_4$ mice compared to Control mice (p=0.007). Yaq-001 administration
1344 was associated with a significant reduction of venous endotoxin compared to untreated-
1345 CCl$_4$ mice (p=0.043).
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1347 **Fig.5. Effect of Yaq-001 on gut permeability in intestinal organoids.**
1348 (A) Intestinal organoids derived and cultured from small intestine of C57BL/6 mice
1349 underwent eversion into apical-out polarity in the first 12 h of suspension culture.
1350 Immunostaining of the microvilli (mv; F-actin) demonstrated that intestinal organoids in
1351 suspension have reversed polarity from basolateral-out to apical-out.
1352 (B) Apical-out intestinal organoids in suspension culture generate goblet cells (MUC2).
1353 (C)Gut permeability of apical-out intestinal organoids was significantly increased by
1354 coculturing with fecal water from CCl$_4$ group than control group (p=0.003). Gut
1355 permeability was notably decreased in fecal water from CCl$_4$-Yaq-001 group compared
1356 to CCl4 group (p=0.001).
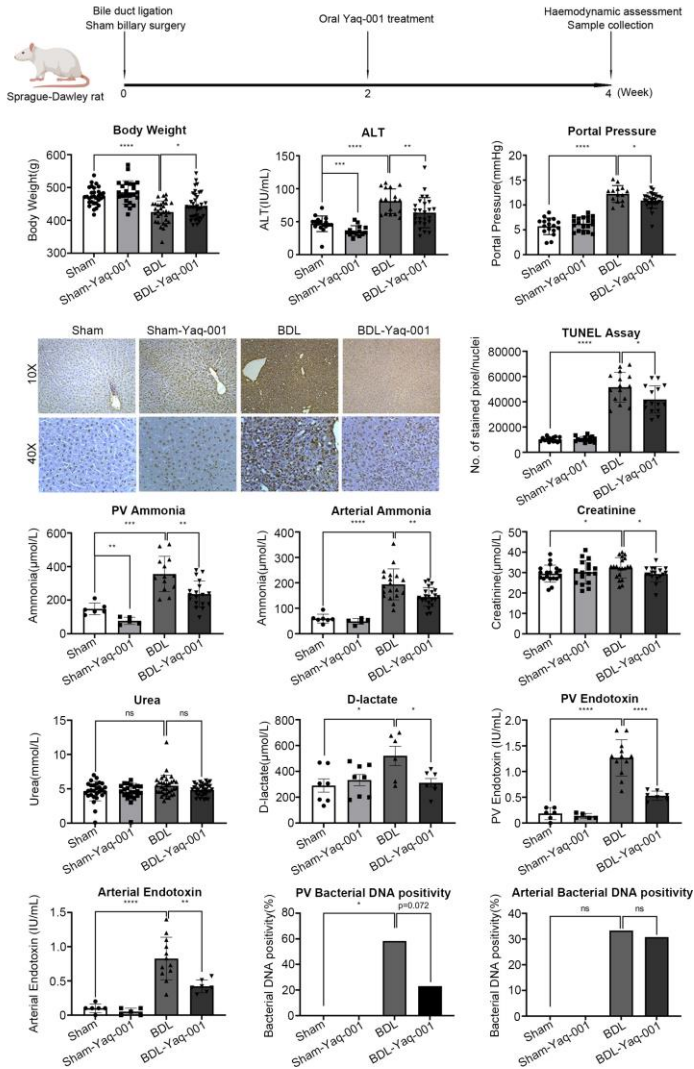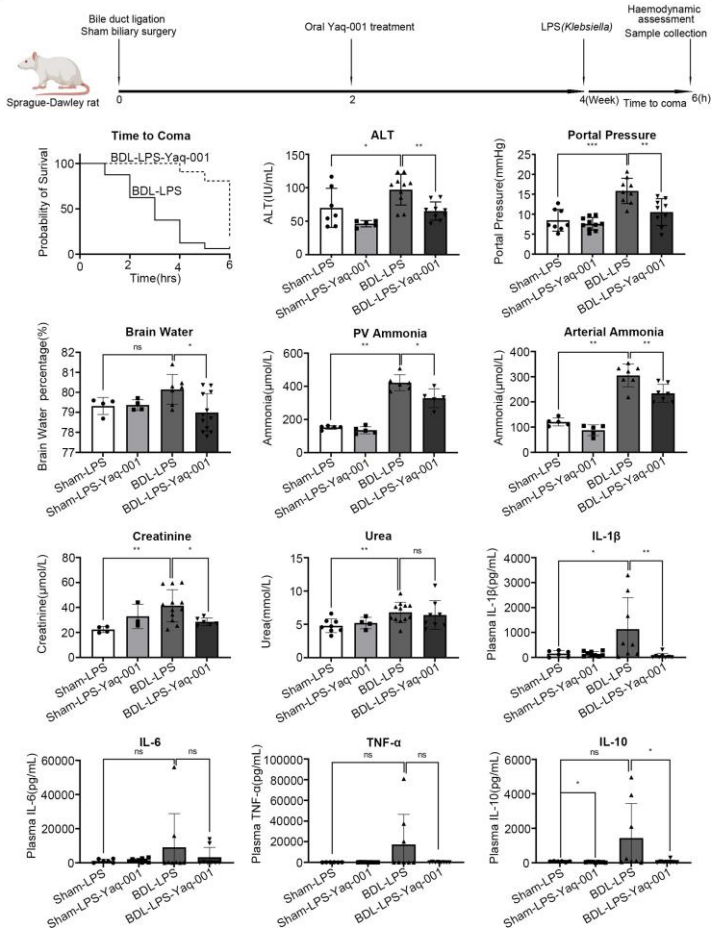1357 (D)Quantification of the integrated density/area of each group.
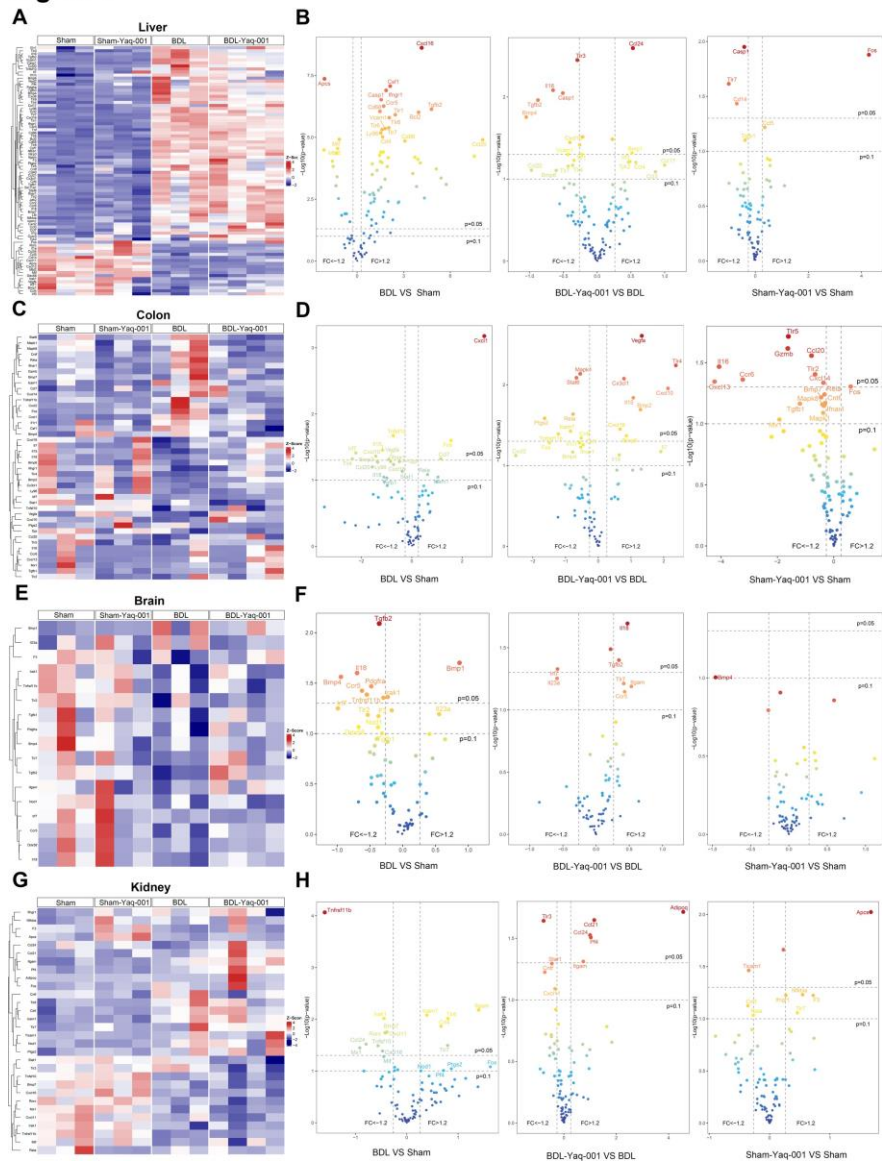1358

Figure 1

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Figure 1

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Figure 2

Figure 3

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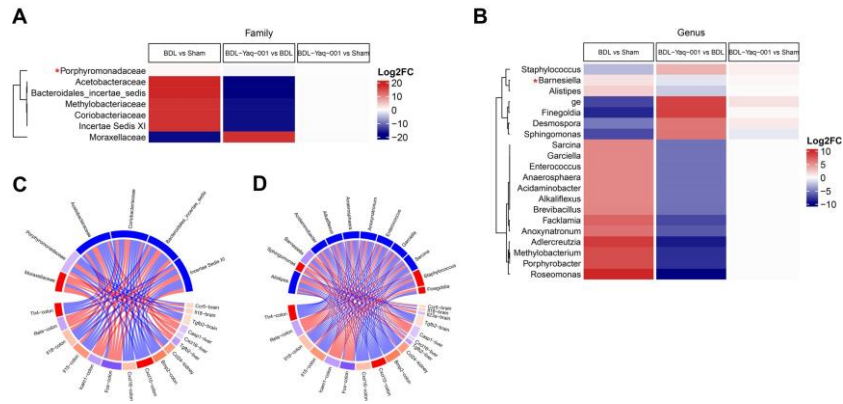
Figure 4

A
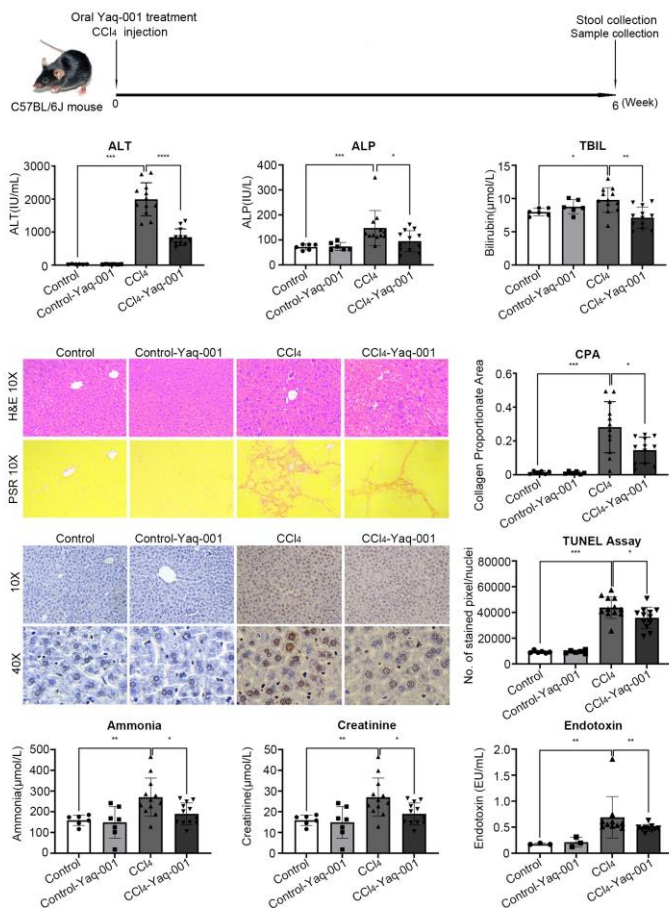
Figure 4

B
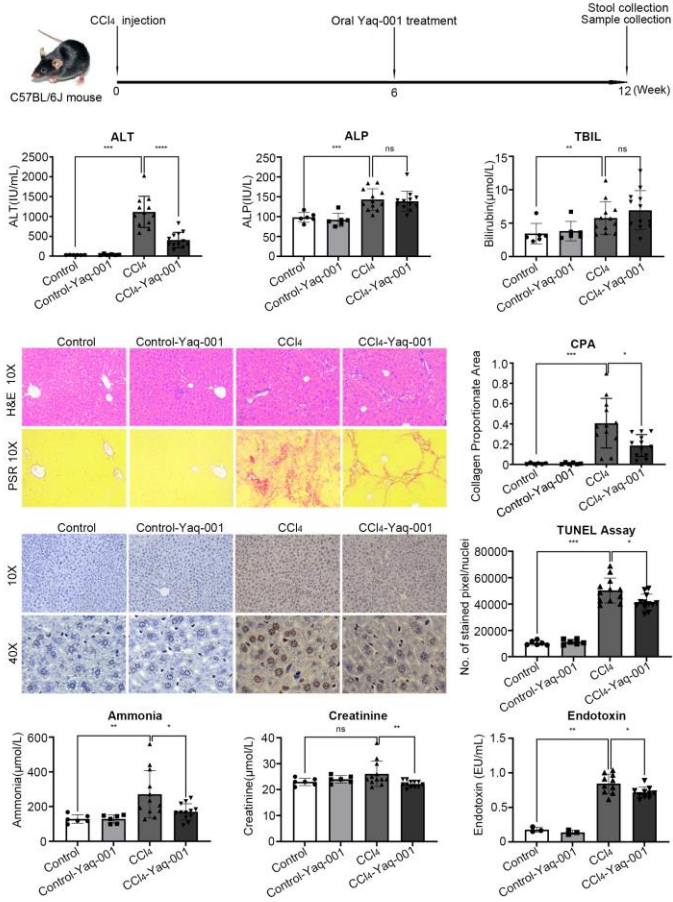
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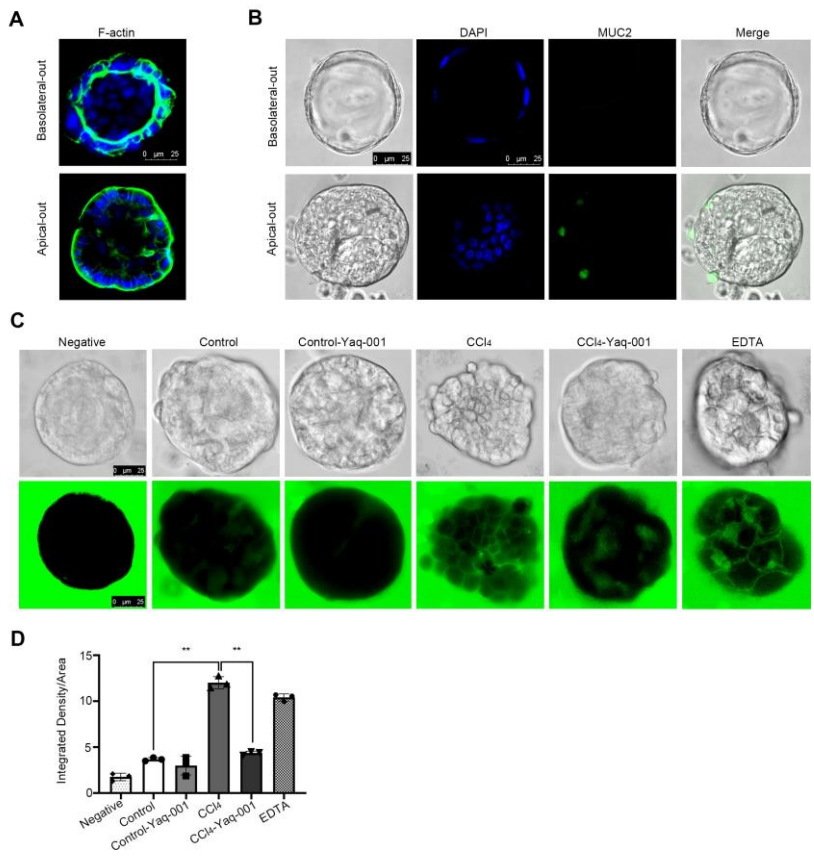
Table 1. Patient Characteristics

	Placebo	Active
Number	12	14
Age(years)	58.5(35-75)	58.5(47-68)
Male sex	9(75%)	10(71.4%)
Race/ethnicity		
Black	0(0%)	0(0%)
Other	12(100%)	14(100%)
BMI (kg/m^2) (SD)	27.8(22.9-32.8)	26.3(20.4-32.8)
Child Pugh Score	7(7-8)	7(7-8)
MELD score	13.2(10.2-16.1)	12.6(9.7-13.5)
Decompensation history		
Alcoholic hepatitis	2(16.7%)	0(0.0%)
Ascites	8(66.7%)	11(78.6%)
Hepatic encephalopathy	5(41.6%)	2(14.3%)
Varices	2(16.7%)	6(42.9%)
Laboratory values		
Bilirubin(μmol/L)	31(19-40)	41(17-68)
Albumin(g/L)	34(30-38)	34(30-39)
Creatinine (μmol/L)	65(53-83)	70(60-81)
Sodium(mmol/L)	136.5(134-137)	137.2(134-140)

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Table 2. Adverse and Serious Events

Adverse Event	Placebo n (%)	Active n (%)
Constipation	3(25%)	2(14%)
Epigastric pain	0(0%)	1(7%)
Nausea	0(0%)	1(7%)
Meteorism	0(0%)	1(7%)
Osophageal reflux	0(0%)	1(7%)
Diarrhoea	2(17%)	0(0%)
Diuresis	0(0%)	1(7%)
Serious Event		
Death	0(0%)	0(0%)
50% increase in MELD	0(0%)	0(0%)
100% increase in creatinine	0(0%)	0(0%)
50% reduction in BMI	0(0%)	0(0%)
Acute decompensation	0(0%)	0(0%)
Episode of ACLF	0(0%)	0(0%)

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Table 3. Safety Parameters: Clinical laboratory assessments, Royal Free Global Assessment and Micronutrient concentrations

Parameters	Placebo			Active		
	Baseline	4 Weeks	12 Weeks	Baseline	4 Weeks	12 Weeks
Laboratory Parameters	Median(range)			Median(range)		
Haemoglobin(g/dL)	122(108-143)	119(107-145)	119(105-139)	122(113-134)	123(113-133)	121(110-137)
Leucocyte count(10^9/L)	4.22(3.47-4.97)	4.05(3.43-4.84)	3.94(3.20-4.90)	5.22(4.20-6.99)	5.12(4.21-6.09)	4.70(4.26-5.52)
Platelets (10^9/L)	81(75-113)	93(83-104)	93(85-100)	95(81-127)	84(75-107)	93(75-105)
Bilirubin(µmol/L)	41(17-68)	31(16-79)	47(20-71)	31(19-40)	42(21-52)	31(17-47)
ALT(IU/L)	25.9(19.88-8.55)	22.89(18.07-31.33)	24.1(18.07-31.33)	25.9(24.1-34.94)	27.11(24.1-33.13)	28.31(19.88-34.94)
ALP(IU/L)	49.2(37.8-52.8)	37.2(24-54)	40.8(27-56.4)	46.8(31.2-51)	46.8(30-69)	45(30-61.2)
Albumin(g/L)	34(30-39)	35(31-40)	32(31-38)	34(30-38)	35(30-38)	32(30-38)
Sodium(mmol/L)	137(135-140)	138(132-140)	138(135-140)	137(134-137)	136(132-137)	137(136-138)
Creatinine (µmol/L)	70(60-81)	77(63-83)	65(63-90)	65(53-83)	64(53-75)	70(57-72)
INR	1.4(1.3-1.6)	1.3(1.2-1.5)	1.4(1.3-1.5)	1.3(1.2-1.4)	1.4(1.2-1.4)	1.3(1.2-1.4)
Child Pugh Score	7(7-8)	7(6-8)	8(6-8)	7(7-8)	8(6.7-9)	8(7-9)

MELD score	12(10-17)	12(10-17)	13(12-16)	13(11-14)	14(11-14)	13(9-15)
MELD Na score	15(10-21)	13(9-19)	16(12-21)	15(13-17)	16(15-17)	14(13-19)
Nutritional Status		**n (%)**			**n (%)**	
Adequate	10(83%)	8(73%)	10(91%)	13(93%)	13(100%)	10(77%)
Moderate malnourishment	2(17%)	3(27%)	1(9%)	1(7%)	0(0%)	3(23%)
Severe malnourishment	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)
Micronutrient Concentrations		**Median(range)**			**Median(range)**	
Vitamin B12(ng/L)	533(290-765)	530(384-970)	524(275-815)	503(337-790)	483(365-694)	462(326-749)
Folate(µg/L)	16(9-22)	17(13-19)	23(21-26)	22(20-30)	20(18-24)	21(17-27)
Vitamin D(nmol/L)	28(20-51)	45(25-57)	43(26-61)	32(25-72)	57(22-72)	70(37-72)
Vitamin A(µmol/L)	0.48(0.13-2.18)	0.47(0.12-1.90)	0.39(0.09-1.80)	0.46(0.15-1.84)	0.39(0.16-1.86)	0.62(0.17-1.82)
Vitamin E(µmol/L)	28.4(17.7-42.0)	32.5(17.6-36.5)	29.9(20.8-36.1)	30.0(19.5-45.6)	30.3(18.3-46.2)	29.4(17.7-37.9)
Vitamin K(µg/L)	0.58(0.16-3.28)	0.33(0.12-2.5)	0.35(0.17-4.2)	0.58(0.14-4.3)	0.41(0.12-5.7)	0.60(0.14-2.8)
Copper(µmol/L)	13.6(8.30-22.6)	13.6(7.10-23.7)	13.5(11.2-24.1)	13.7(10.0-30.1)	15.5(9.10-26.7)	15.1(7.80-37.6)

Zinc(µmol/L)	9.10(5.60 -15.3)	8.85(5.00- 13.7)	8.40(5.70 -14.3)	8.20(5,6 0-12.9)	8.60(4.70 -15.0)	9.00(5.00- 15.8)
Selenium(µmol/ L)	0.78(0.56 -1.10)	0.86(0.47- 1.12)	0.90(0.65 -1.04)	0.77(0.5 5-1.14)	0.93(0.48 -1.23)	0.91(0.49- 1.46)